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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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TITLE OF INVENTION (280 characters maximum)

TRANSMUCOSAL ADMINISTRATION OF AGGREGATED ANTIGENS

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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*check one*)

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October 17, 2002
(Date)

Respectfully submitted,
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[ ] Additional inventors are being named on separately numbered sheets attached hereto.

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PTO/SB/16 (8-00)

TITLE

Transmucosal Administration of Aggregated Antigens

BACKGROUND OF THE INVENTION

The mucosal immune system is defined herein as those immune cells and organs directly associated with the mucosal lining of the gastrointestinal tract and lungs, including the airways. Such a partitioning of the body's total immune network is not arbitrary, but is based on certain widely recognized unique features of the mucosal immune system.

One of the distinguishing features separating mucosal immunity from other types of immunity is the requirement that the mucosa physically transports, or permits ingress of foreign substances into the body. In the gut, nutritional substances must be absorbed for metabolic purposes. Many, and probably most of the absorbed nutrients are antigenic. Proteins, lipoproteins, carbohydrates complexed with proteins, and the like can and do stimulate immune priming and subsequent recognition. Further, because of the large concentration of symbiotic (but potentially pathogenic) microorganisms in the gastrointestinal tract, the mucosal immune system must prevent adverse immune reactions that would result in elimination of the symbionts through inflammatory immune recognition.

Another distinctive feature of the mucosal immune system is the ability to generate a specific isotype of antibody (IgA) which can prevent absorption of antigen but is non-inflammatory. While certain sub-classes of IgG are similarly non-inflammatory, IgA is predominantly localized to the mucosal immune system.

It is also recognized by those skilled in the art, that the mucosal immune system is the numerically dominant member of somatic immunity. Studies have shown that each meter of human intestine contains approximately 10^{12} lymphocytes. On the basis of immunoglobulin-secreting cells, the human gut contains several-fold more cells than the remainder of the immune system. Additionally, the phenotypic character of the immunocytes populating the mucosal immune system is distinctive. Murine and human intestine contain a large percentage (up to 90%) of an atypical lymphocyte; the gamma-delta T lymphocyte. These cells are unique because they are not educated in the thymus

during ontogeny as are the majority of alpha-beta T cells populating the spleen, peripheral lymph nodes, or circulation.

Yet another feature of the mucosal immune system that separates it from the somatic immunoaxis is the presence of predominantly anti-inflammatory T helper 2 cytokines. The tissue supporting the single-cell barrier separating the lumen of the gastric or respiratory system from the external milieu, the lamina propria, is rich in interleukin-4, interleukin-10, and transforming growth factor-b. It is known to those skilled in the art that immune cells antigenically stimulated in such an environment differentiate into immune effectors with anti-inflammatory (TH2) functions. However, such an environment does not guarantee ultimate TH2 function, and this has been shown to occur in the mucosal immune system. Specifically, antigen presentation to the mucosal immune system typically has three outcomes as set forth below.

1. When an antigen is transported across the mucosal epithelium, it can be processed by immune accessory cells, which may migrate to local immune accessory organs (regional lymph nodes) and there cognitively present antigen or antigen fragments to other immune cells resulting in the numerical expansion of antigen-reactive lymphocytes. A proportion of the activated lymphocytes mature to plasma cells that manufacture the mucosal-dominant, non-inflammatory immunoglobulin IgA. Another proportion of the cells differentiates into inflammatory (TH1) cells such as IgG2a secreting plasma cells, interferon-gamma or Interleukin-2 expressing T helper lymphocytes.

2. Some of the processed antigen is presented to immature B lymphocytes that differentiate into IgA secreting plasma cells. These cells migrate back to the mucosa where they establish residence, and express the specific immunoglobulin. The majority of the immune protein is then transported into the lumen of the gut or lungs where it reacts with epitopes of the educating antigen. Resultant antibody binding to the immunogen can prevent transmucosal ingress.

3. The mucosal immune system, perhaps due to its unique physiological responsibilities, concomitantly drives one of two additional immunological events. These events are not mutually exclusive, and are believed to occur in a continuum dictated by the amount of antigen administered as well as dose timing, frequency, and simultaneously

administered proteins. The first of these events is the education and expansion of another arm of the immune system (low dose tolerance).

When small (ca. unit milligram) amounts of the antigen are presented to the mucosa, a set of lymphocytes is enabled for education and expansion. The distinguishing characteristic of this subset of educated cells is their ability to accept a biochemical signal of active inflammation and recognize the presence of the original educating antigen in intimate physical proximity to sites of inflammation. When both activation criteria are present, the suppressor lymphocytes express certain immune regulatory cytokines resulting in an active down regulation of the inflammatory reaction. These biochemicals include interleukin-4, interleukin-10 and TGF-B.

A second mechanism of tolerance engendered by antigen presentation to the mucosal immune system is referred to as "high-dose tolerance". This mechanism comes into play following administration of larger (ca. 10 mg or larger) amounts of the educating antigen. "High dose" tolerance appears to directly disable the antigen-reactive lymphocyte. It has been suggested that the administration of large amounts of antigen results in a reduced proteolytic processing of the antigen in the gut or lungs with an increased concomitant availability of non-degraded antigen which is transferred across the mucosal barrier intact. Reactive lymphocytes bind large amounts of the antigen, and are rendered anergic perhaps because the stoichiometry of antigen presentation with required activation signal, although other explanations have been offered. Experimentally, the two mechanisms are differentiated by the demonstrable requirement for educating antigen and presence of IL-4, IL-10, and TGF-B in immunosuppressed cultures or animal models in the first instance. In contrast, anergy is experimentally demonstrated by reversal of immune unresponsiveness by the addition of exogenous IL-2, and the relative paucity of suppressive cytokines present in active suppression.

Published work from several laboratories has confirmed that both of the above-described mechanisms may operate concomitantly. The foregoing discussion is provided to clarify the known mechanisms of oral tolerance, and not to differentiate them. Both operate to suppress immune responsiveness to mucosally presented antigen, and appear to be functional in the suppression of inflammatory responses to inhaled or eaten foreign substances.

Virtually all mucosally presented antigens elicit tolerance. Low dose tolerance occurs following the administration of myelin basic protein in a model of multiple sclerosis, type II collagen in a model of rheumatoid arthritis, retinal S antigen in a model of autoimmune uvitis and insulin in a model of type I diabetes. Further, mice fed recombinant acetylcholine end plate receptor protein have been found to be refractory to immune stimulation with the same protein in a model of myasthenia gravis. Additionally, oral ovalbumin administration has been shown to elicit tolerance in ovalbumin-TCR transgenic mice. High dose tolerance has also been reported for some of the same as well as different proteins.

There are certain reported exceptions in the otherwise universal ability of orally or nasally administered proteins to establish oral tolerance. These exceptions may be divided into two categories based on either the biochemical properties of the administered protein or the physical form of the antigen. The oral administration of cholera holotoxin resulted in systemic immunity based on several experimental criteria. Similarly, the mucosal exposure of mice to the heat labile enterotoxin of certain *E. coli* strains resulted in the presence of circulating IgG antibodies reactive with the protein. Both of these substances belong to a unique class of toxins that are known to activate phosphorylation of specific transmembrane receptors present in enterocytes in the mucosa. Additionally, the diseases caused by the microorganisms that produce these proteins are enteric, and thus, the proteins are known to be naturally pathogenic at the mucosal level.

The second form of exception to orally administered proteins inducing an immune response instead of tolerance concerns the physical form of the presented antigen. Arntzen and colleagues reported the presence of systemic antibodies reactive with the nucleocapsid of Norwalk virus after feeding extracts of tobacco containing the transgenically expressed virus product (Arntzen et al.,1996, PNAS 93(11):5335-40). These researchers also discovered the presence of systemic immunity to hepatitis B surface protein (HBsAg) in mice fed repeated doses of following isolation of the protein expressed in a similar transgenic plant system (Kong et al., 2001, PNAS 98(20):11539-44.). However, the simultaneous administration of the mucosal adjuvant cholera holotoxin was required for maximal effect. A minor response was induced by feeding

primed mice the plant extract alone, perhaps due to the physical form of the antigen. Importantly, apparently intact 17 nM pseudovirions were seen in homogenates of the plants, indicating that the transgenic protein self-assembled within the plant tissues

Another example of the ability of orally presented antigens to elicit an immune response is found in the recent work of Koprowski and colleagues. These investigators inserted the coding sequences for two protective epitopes from the rabies virus into alfalfa mosaic virus coat protein (CP), and then rescued the transgenic protein by enabling viral replication in plants in a complemented system using either tobacco mosaic virus lacking native CP(A4-g24), or in trans following infection with infectious alfalfa mosaic virus. Mice fed the chimeric viral particles were found to develop a systemic immune responsiveness (Yusibov et al., 2002, Vaccine 20(25-26):3155). Koprowski et. al. engineered a commercial cultivar of tomato to express the coat protein of the rabies virus, and reported that mice fed freeze-dried fruit became immune to challenge with normally lethal concentrations of the virus (Kapusta et al., 2001, Adv. Exp. Med. Biol. 495:299-303). Additional work by these researchers provided evidence that tomato's expressing the HBsAg could elicit circulating antibodies in mice formally adequate to provide immune protection from infection based on established IgG concentrations in humans (Kapusta et al., 1999, FASEB J. 13(13):1796-9). These results, collectively, suggest that oral administration of certain vaccine antigens results in the boosting of immunologically primed animals, or in rare cases the establishment of protective immunity in immunologically naive mice, but not the anticipated immune indolence of mucosal tolerance. An important common feature of these results is that the transgenically expressed antigen was either biochemically constrained to assemble into macromolecular structures, or was detected as complexed with plant organelles such as Golgi bodies, vesicles, plasma lemma and cell walls; structures effectively forming macromolecular complexes.

There is a long felt need in the art for efficient methods of immunizing a patient against an antigen. There is also a need for a method of presenting antigens to efficiently induce mucosal tolerance. The present invention meets these needs.

SUMMARY OF THE INVENTION

The present invention includes a method of inducing a systemic immune response to a peptide in a mammal, wherein one of the method steps involves transmucosally administering to the mammal a macromolecular aggregate of the peptide, thereby inducing a systemic immune response in the mammal.

In an aspect of the invention, a method of inducing an immune response includes administration of a macromolecular aggregate of at least 10 peptide subunits, and more preferably, at least 20 subunits. In another aspect of the invention, the macromolecular aggregate may have a molecular weight in excess of 1,000 kD.

In one aspect of the present invention, a method of inducing an immune response includes administration of a macromolecular aggregate of at least 1 nm in diameter, and more preferably, at least 5 nm in diameter.

In an embodiment of the present invention, a method of inducing a systemic immune response involves administering a macromolecular aggregate resistant to digestive degradation. In one aspect of the invention, a macromolecular aggregate is resistant to digestive degradation through stabilization in aggregate form by chemical treatment. In another aspect of the invention, the macromolecular aggregate is stabilized in aggregate form by recombinant protein engineering of the peptide. In yet another aspect of the invention, the macromolecular aggregate of a peptide stabilized in aggregate form by recombinant protein engineering is further stabilized in aggregate form by subsequent chemical treatment.

In an embodiment of the present invention, a method of inducing an immune response includes administration of a macromolecular aggregate of a protein derived from hepatitis B. In one aspect of the invention, the macromolecular aggregate is an aggregate of hepatitis B viral surface protein. In another aspect of the invention, the macromolecular aggregate is an aggregate of hepatitis B viral nucleocapsid protein. In yet another aspect of the invention, the macromolecular aggregate is an aggregate of hepatitis B viral envelope protein.

An embodiment of the present invention provides a pharmaceutical composition for inducing systemic immunity in a mammal, wherein the pharmaceutical composition includes a macromolecular aggregate of a peptide and a suitable

pharmaceutical carrier, in an amount sufficient to induce systemic immunity when administered to a mammal transmucosally.

In another embodiment of the invention, a method of suppressing a systemic immune response to a peptide in a mammal already immune to that peptide is provided. Such a method of suppressing a systemic immune response in a mammal includes transmucosal administration of a macromolecular aggregate of the peptide to a mammal in order to suppress a systemic immune response.

In an aspect of the invention, a method of suppressing an immune response includes administration of a macromolecular aggregate of a peptide composed of less than 20 subunits of the peptide. In another aspect of the invention, the method of suppressing an immune response includes administration of a macromolecular aggregate of at less than 1 nm in diameter.

In an embodiment of the present invention, a method of suppressing a systemic immune response involves administering a macromolecular aggregate resistant to digestive degradation. In one aspect of the invention, a macromolecular aggregate capable of suppressing a systemic immune response is resistant to digestive degradation through stabilization in aggregate form by chemical treatment. In another aspect of the invention, the macromolecular aggregate is stabilized in aggregate form by recombinant protein engineering of the peptide. In yet another aspect of the invention, the macromolecular aggregate of a peptide stabilized in aggregate form by recombinant protein engineering is further stabilized in aggregate form by subsequent chemical treatment.

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An embodiment of the present invention provides a pharmaceutical composition for suppressing systemic immunity in a mammal, wherein the pharmaceutical

composition includes a macromolecular aggregate of a peptide and a suitable pharmaceutical carrier, in an amount sufficient to induce systemic immunity when administered to a mammal transmucosally.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1 is the DNA sequence of the HBcAg gene (SEQ ID NO:1) and the amino acid sequence for the corresponding protein (SEQ ID NO:2) for HBcAg from HBV serotype ayw. The sequence was obtained from the National Library of Medicine.

Figure 2 is a graph illustrating that the oral administration of ag HBcAg to previously immunized mice induces suppression of the proliferation of antigen challenged immune spleenocytes in culture. Column 1 indicates whether the mice were immunized, boosted and orally tolerized as described. Column 2 depicts the response of spleen cells from mice sham tolerized with PBS and antigen challenged. Columns 3 through 5 represent the proliferation of spleen cells from animals tolerized with 0.0,.1, 1 and 10 of ag HBcAg, respectively.

Figure 3 is a graph illustrating that Con A stimulated spleen cells from normal mice proliferated equally in the presence or absence of native HBsAg at various concentrations. Briefly, spleen cells from a normal mouse were collected and placed in culture. Con A (5 μg/well) was added and serial dilutions of native HBsAg was added. The concentrations ranged from 0 μg/well to 20 μg/well in 2-fold dilutions. Proliferation was determined as described.

Figure 4 is a graph illustrating that oral administration of native HBsAg boosts the immune response of antigen-challenged spleen cells from immune mice. Briefly, mice were immunized with the native antigen, boosted, and some of the mice were then orally tolerized with native HBsAg (pseudovirions) at the concentrations indicated. Following treatment, the mice were euthanized, and then the spleen cell

suspensions were prepared and challenged with the appropriate antigen. Proliferation of the cells was quantified by ^{3}H-Tdr incorporation and scintillography.

Figure 5 is a graph depicting the local presence of immune reactive HBsAg at varying densities (sizes) after fractionation on a sucrose density gradient following isopycnic ultracentrafugation. HBsAg was disrupted with 2-mercaptoethanol, 8 M urea and heat. The lysate was applied to a Sepharose G-200 column and fractions were collected while monitoring the OD_{280nm}. Each fraction was then tested for the presence of immunoreactive HBsAg. Fraction numbers are on the X-axis and OD_{280nm} measurements are on the left Y-axis. The OD of the ELISA assay for each fraction is presented on the right Y-axis.

Figure 6 is a graph illustrating that chemical dissociation of native HBsAg restores the tolerance induction capacity of the antigen. Mice were immunized and boosted as described in Example 5, and some mice were orally tolerized with Pool 1 native antigen (set 2), Pool 1A disrupted antigen (set 3), Pool 2 native HBsAg (set 4) Pool 2A (disrupted HBsAg) (set 5), Pool 3 (Native antigen) or Pool 3A (disrupted HBsAg). Spleen cells were processed for culture, and all cultures challenged with 10 μg of native HBsAg with the exception of set 1 which received no antigenic stimulus. The left axis presents proliferation results measured as CPM.

Figure 7 is a bar graph illustrating that HBeAg is superior to either native HBcAg or aggregated HBcAg as a tolerogen on a per-weight basis. For four treatments every second day, mice were immunized with ag HBcAg, boosted, and then 28 days later, orally tolerized with PBS or the indicated amounts of one of the physical forms of HBcAg explored herein. The left (horizontally striped) bars represent the proliferation response when spleen cells from immune mice challenged with native HBcAg in vitro. The right (vertically striped) bars represent the results of spleen cells collected from immune and orally tolerized mice. Cells were challenged with 5 ug of ag HBcAg per well. The left axis presents the incorporated ^{3}H-Tdr as CPM.

Figure 8 is a line graph showing the size distribution of ag HBcAg and the local presence of immune reactive ag HBcAg at varying densities (sizes) after fractionation on a sucrose density gradient following isopycnic ultracentrafugation. Ag HBcAg was applied to a Sepharose G-200 column and were fractions collected while

monitoring the OD at 280 nm. Each fraction was then tested for the presence of immunoreactive ag HBcAg. Fraction numbers are on the X-axis and OD_{280nm} measurements are on the left Y-axis. The OD of the ELISA assay for each fraction is presented on the right Y-axis.

DETAILED DESCRIPTION OF THE INVENTION

Foreign substances presented to the mucosa of the gastrointestinal tract or the lungs act as immunogens. Because these mucosal surfaces are unit cellular thickness and have the physiological responsibility of absorption, foreign substances are known to elicit immune stimulation. To prevent adverse immune reactions in the host, a physiological compensatory mechanism has evolved. This mechanism is alternatively called oral or mucosal tolerance. Two forms of mucosal tolerance are known to those skilled in the art. In the case of "low dose" tolerance, antigens eliciting an immune response concomitantly elicit educated lymphocytes capable of suppressing inflammatory immune reactions upon experiencing specific stimuli consisting of A) at least one biochemical signal of inflammation and B) the educating antigen in intimate physical proximity. The second mechanism, "high dose" tolerance results in anergizing inflammatory cells. The two mechanisms are not mutually exclusive, and may both operate in an individual at the same time. Both forms of tolerance are useful for treating certain diseases in animals, including humans.

In one preferred embodiment, a class of diseases, termed autoimmune diseases, may be attenuated or suppressed by the oral or by inhalation administration of antigens known to be among the immune targets attacked by an individual's immune system. This therapeutic application is described by the teachings of Weiner and associates in U.S. Patent Numbers 6077509, 6039947, 6036957, 6019971, 5961977, 5935577, 5869093, 5869054, 5858980, 5858968, 5858364, 5856446, 5849298, 5843886, 5843445, 5840848, 5807993, 5783188, 5788968, 5783188, 5763396, 5733547, 5720955, 5681556, 5643868, 5641474, 5641473, 5593698, 5571500, and 5571499.

In yet another preferred embodiment of the therapeutic approach, the disease following certain chronic infections of animals may be attenuated or suppressed by mucosal tolerance therapy as taught in U.S. Patent No. 6,355,248, wherein the

disclosed invention uses physiochemical forms and compositions of the tolerogens used to engender mucosal tolerance for the latter preferred embodiment.

The dichotomy of oral tolerance induced by most fed or inhaled antigens versus the induction of systemic immunity following the oral presentation of other antigens is resolved by the present invention. Specifically, the present invention teaches that the physical form of the protein administered dictates which natural course ensues following mucosal administration of the antigen. More specifically, the present invention teaches that proteins that are biochemically constrained to assemble into macromolecular aggregates of greater than approximately 10, but preferably about 20 subunits, or which approach physical sizes greater than 1 nm, but preferably greater than 5 nm in the largest diameter engender selective uptake by M cells of the Peyer's patches of the intestine resulting in systemic immunity. Antigens that are expressed as monomolecular species or those that are engineered or physically/chemically treated to prevent macromolecular assembly engender oral tolerance instead. This is important because the generation of immunity following oral presentation of antigen may exacerbate disease in patients with chronic infections and treated with mucosal tolerance induction as taught in U.S. Patent No. 6,355,407.

Additionally, the present invention teaches that assemblies of monomer subunits that are resistant to digestive degradation also enhance the development of systemic immunity as opposed to mucosal tolerance. The present invention is also useful in that the formulation of the composition of antigen may be specifically directed to induce oral tolerance. The invention is also useful for the design and composition of therapeutic agents for the treatment of a subset of chronic infections described in U.S. Patent No. 6,355,407.

Definitions

The term "recombinant protein engineering" is used herein to denote a genetic or molecular biology-based method of altering the nucleic acid sequence encoding the naturally-occurring amino acid sequence of a protein. Such a method may employ one or more of a random, iterative, calculated, rational design, or molecular modeling-based technique in order to mutate, add, or subtract amino acid residues from

the naturally-occurring amino acid sequence of a protein for the purpose of altering the native properties of the naturally-occurring protein. Such properties include, but are not limited to, thermal stability, pH stability, stability as a function of ionic strength, primary-, secondary-, tertiary-, or quaternary structure, oligomeric state, and interaction with accessory proteins or cofactors.

As used herein, "modulating an immune response of a mammal" means increasing or decreasing either the amount of a component of the immune system or the activity by which a component of the immune system is characterized. By way of example, modulating an immune response of a human includes increasing the number of suppressor T lymphocytes present in the human, increasing secretion of immunosuppressive factors by a suppressor T lymphocyte in the human, decreasing the number of cytotoxic T lymphocytes present in the human, decreasing the cytotoxic activity of a cytotoxic T lymphocyte in the human, decreasing the amount of an antibody in the human, decreasing the amount of a complement protein in the human, decreasing the ability of a complement protein to interact with a cell in the human, and the like.

As used herein, an "epitope" means a molecule or a portion of a molecule which interacts or is capable of interacting with an immunoglobulin molecule produced by the immune system of a mammal such as a human. An antigen is a well known example of an epitope which is capable of interacting with an antibody. It is understood that a single molecule may comprise numerous epitopes, and that an epitope may comprise a portion of each of more than one molecule.

An "epitope located in close proximity to the immune response" means an epitope present on the surface of at least one cell of a tissue located at a site of undesirable immune reactivity, wherein the reactivity is induced or exacerbated by the presence in the mammal of the infectious agent, or an epitope which is cross-reactive with such an epitope. By way of example, the presence of the hepatitis B virus (HBV) induces the human body to produce cytotoxic T lymphocytes which attack hepatic cells that display a viral protein comprising a particular epitope on their surface. In this case, both production of these T lymphocytes and the cytotoxic activity of these T lymphocytes toward hepatic cells are undesirable immune reactivities. By transmucosally administering to an HBV-infected human a composition comprising the same or a similar

epitope, immunosuppressive lymphocytes such as suppressor T lymphocytes are produced by the body. These lymphocytes are capable of migrating to the hepatic tissue which displays the epitope and of suppressing the cytotoxic activity of T lymphocytes produced in response to the presence in the human of HBV, thereby modulating the undesirable immune reactivity.

A "macromolecular aggregate" of a peptide as used herein describes a physical association of two or more units of the individual peptide molecule. The peptide molecule may associate specifically or non-specifically. By way of a non-limiting example, the "non-specific macromolecular aggregation" of a peptide according to the present invention may involve an ionic- and/or hydrophobic-based interaction of peptides effected by either increasing or decreasing the salt concentration of a peptide solution. By way of another non-limiting example, a "specific macromolecular aggregation" of the present invention may involve a rationally-designed amino acid mutation (or multiple amino acid mutations) that allows the mutated peptide to specifically associate with other similar mutated peptides in a controlled manner. Specific association by way of the latter example may also be effected, enhanced, or decreased with the assistance of chemical compounds known by one of skill in the art to be useful in modulating peptide and protein interactions.

EXPERIMENTAL EXAMPLES

The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1: Induction of Mucosal tolerance by aggregated hepatitis B virus core antigen (HBcAg).

Construction, expression, purification and physical characterization of aggregated HBcAg (ag HBcAg). The plasmid pTKHH2 containing a head to tail dimer of the complete genome of hepatitis B virus was treated with EcoR1 endonuclease to

cleave at the boundaries of the genome. The resulting digest was phenol/chloroform extracted, and electrophoresed into a 0.5% agarose gel. The 3182 base hepatitis B virus (HBV) genome band of DNA was collected, and the virus core gene was excised using EcoR1 and HindIII. Following phenol/chloroform purification, the product was ligated into the multiple cloning site of pETb 28 (NovaGen). Competent E coli. BL21 cells (NovaGen) were transfected with the plasmid, and colonies were selected based on their resistance to kanamycin. Several colonies were expanded, and minipreps were performed to extract the plasmids. Several plasmids were sequenced and were determined to contain the authentic coding region of the hepatitis B virus core gene. One colony was selected based on its relative expression of HBcAg as determined by western dot blot assay. This clone was expanded in selection broth and the DNA isolated as described previously. A sequence encoding 6 histidines and the T7 peptide was inserted 3' to the HBcAg gene (pET28A vector system, NovaGen) and transformants selected as described. The transgenic protein was expressed by inoculating 50 ml of LB broth with the transgenic bacteria and incubating at 37°C overnight with shaking. The following morning, the culture was diluted 10 fold with fresh LB containing antibiotics, and when the OD_{600nm} reached approximately 0.5, adequate isopropylthiogaloctopyranaside (IPTG) was added to make the culture 1 mM in IPTG. After 2 hours, bacteria were collected by centrifugation, and the pellets were washed once with cold PBS. Bacterial pellets were then resuspended in 8M urea at pH 8.0 and the debris was cleared by centrifugation. The lysed culture was then applied to a column containing Ni-NTA agarose (Qiagen Corporation). The column was washed with 10 volumes of wash buffer as recommended by the supplier, and the product was eluted using a pH 4.5 buffer. Following dialysis against PBS, the purity and identity of the protein was established by PAGE electrophoresis followed by silver staining and western blotting. This protein was found to self-aggregate following dialysis in PBS, yielding a flocculent suspension, and is referred to hereinafter as aggregated HbcAg, as opposed to native HBcAg which assembled into pseudocapsids (Schodel et al., 1993, JBC 268:1332-1337) or modified HBeAg as described elsewhere herein.

Tolerance induction in mice

CB17, C3H and C57 BL/6 mice of both sexes were used in these

experiments. All animals were between 6 and 14 weeks of age. Mice were immunized with 50 μg native HBcAg particles (nHBcAg) emulsified in an equal volume of Complete Freund's Adjuvant (CFA) on day 36, and received an intraperitoneal booster of HBcAg on day 14.

Control animals were sham inoculated with PBS. On days 2, 4, 6 and 8, selected animals were fed 1 mg or 5 mg of test protein (nHBcAg, aggregated HBcAg or HBeAg as indicated), solubilized in between 0.25 and 0.5 ml of PBS, with a ball point feeding needle. Control animals received an equal volume of PBS. Between days 10 and 17, animals were euthanized and the spleen cells collected and placed in culture in replicates of five. Cells were diluted to contain either $1x10^5$ or $2x10^5$ splenocytes per well in 100 μl of Iscove's medium containing 10% FCS, 50 μg /ml penicillin, 50 μg /ml streptomycin, and $5x10^{-5}$ 2-mercaptoethanol. After 72 and 96 hours, each well received 1 μCi tritiated thymidine, and the incorporated label quantified by scintillography.

Spleen cells collected from immune mice (Immune), or from mice immunized then orally tolerized (Immune/OT) did not significantly proliferate in the absence of antigen in the cultures. When 1 μg per well of HBcAg was supplied, all cells proliferated, but cells from Immune/OT mice underwent mitosis at a significantly lower level. In cultures to which 5 μg of HBcAg had been added, the level of cellular replication was greater still, and the cells isolated from Immune/OT mice again exhibited a significantly depressed proliferation compared to mice not orally fed antigen. Clearly, mucosal presentation of the immunizing antigen after immunity had been established resulted in a dose dependant suppression of the immune response. Data shown are representative of results of at least 6 experiments (Figure 2).

Example 2: In vitro toxicity of HBsAg

Another HBV protein as tested for its ability to induce oral tolerance in alternate physical forms. Recombinant hepatitis B surface antigen existing in 25 nM spheroids (pseudovirions) was purchased from Korea Green Cross. This protein is sold as a vaccine in many countries and has been extensively characterized. It was possible that the recombinant HBsAg obtained might contain contaminants that would adversely affect the growth of immune cells. To test this, the protein was serially diluted to provide

concentrations from 3 μg/5μl to 0.01 μg /5 μl, and was added to replicates of cultures of spleen cells collected from normal mice. Control wells received Con A but no antigen. Cells were stimulated with 5 μg /well of Con A, and were cultured for 4 days. Radioactive thymidine was added to each well, and the cells were cultured for an additional 18 hours. Cells were collected, and their proliferation was quantified by scintillography (Figure 3).

No statistically significant differences were observed between culture replicates suggesting that the HBsAg was neither toxic nor did it interfere with the proliferation of spleen cells stimulated with a mitogen.

Example 3: Induction of Mucosal tolerance by HBsAg.

Hepatitis B surface antigen (HBsAg) was purchased from Korea Greencross vaccines (Jong Jin City, South Korea). This protein preparation has been shown to consist of predominantly 25 nm spheroids comprised of about 100 subunits of the small HBsAg protein. This particular protein was used in all phases of the HBsAg-requiring experiments. CB17 mice received immunizations of 50 μg of the protein emulsified in Complete Freunds Adjuvant on day 28. All animals received 10 μg of the same protein in PBS as intraperitoneal boosters on day 14. On days 1, 2, 4 and 6, indicated animals were fed 0.1, 1 or 5 mg doses in 0.25 ml of PBS using a ball-tipped feeding needle. Control animals received an equal volume of PBS. The treatments were administered every second day for a total of 4 doses. On day 10, animals were euthanized, and the spleens were aseptically collected. Single cell suspensions were prepared, and dispensed into replicate wells of a 96 well tissue culture cluster. Varying doses of the antigen were added, and the cultures were maintained for 72 hours in 5% CO_2/balance air. Each well then received 1 μCi of tritiated thymidine and were cultured for an additional 18 hours. The cells were then collected onto glass fiber filters. Incorporated label was determined by scintillography.

The results of this experiment clearly showed that the oral administration of either of two concentrations of the viral protein resulted in enhanced immune reactivity to the protein in immune animals (Figure 4). This is in direct contradiction to the preceding experiment in which oral administration of a viral protein resulted in less

immune reactivity to the antigen in vitro. To determine whether the physical conformation of the administered antigen was responsible for the booster effect observed, the HBsAg was disrupted by detergent and disulfide bond scission, rapidly renatured, and then tested again for its ability to induce mucosal tolerance.

Example 4: Physiochemical disruption of HBsAg pseudovirions

To disrupt the 25 nm HBsAg pseudovirions, an aliquot of the protein was dialyzed against PBS containing 2% SDS and 2% 2-mercaptoethanol overnight at 5°C then heated to 60°C for 1 hour. The denatured protein was layered on top of a discontinuous sucrose density gradient consisting of 50%, 40%, 30%, and 20% sucrose in PBS, and then centrifuged for 13 hours at 35,000 RPM in a Beckman SW41 rotor. An aliquot of the non-disrupted native antigen was identically processed. Fractions of 0.30 ml were collected, and optical density at 280 nm and presence of immunoreactive proteins determined for each. Specifically, column eluates were monitored with an flow-through cell mounted in a spectrophotometer adjusted for 280 nm. Immunoreactive protein was determined by transferring 10 µl of each fraction to duplicate wells of a 96 well ELISA plate cluster (Nuncleon), and allowing overnight adhesion at 5°C. Wells were washed with PBS, and unoccupied protein binding sites saturated with 100 µl of 5% powdered milk dissolved in PBS. Following 3 washes with PBS, each well received 50 µl of a 1:5000 dilution of polyclonal rabbit anti-HBsAg for 45 minutes at room temperature. Following washing, all wells were treated with a 1:5000 dilution of goat anti-rabbit Ig that had been conjugated with horseradish peroxidase (Sigma). After an additional 45-minute incubation, all wells were washed with PBS, and 50 µl of 2, 4-orthophenylamine diamine/H_2O_2 solution (Sigma) was added, and the optical density of each well determined with a microplate ELISA reader at 492 nm. Replicate wells were averaged, and the results were plotted (Figure 5).

These experiments clearly suggested that the physical size and/or conformation of the protein present in the native preparation, as detected by OD_{280nm} protein assay, equilibrated in the denser portions of the gradient. In contrast, proteins recognized by the rabbit antibody specific for HBsAg were present in the lighter

fractions. Further, HBsAg disrupted with 2-ME and SDS presented as several immunoreactive peaks including a major band in the lightest fractions.

Example 5: Induction of mucosal tolerance by disrupted HBsAg

A 0.5 cm x 15 cm chromatography column was loaded with Sephadex G200 (Sigma), which can resolve globular proteins ranging between 20 KD and 200 KD. Approximately 14 mg of denatured HBsAg was applied to the column, and proteins were eluted with PBS. A total of 48 fractions of 0.25 ml each were collected. The protein concentration and immunoreactivity was determined for each, and the fractions were combined into three pools. Pool 1 contained the equivalent of fractions 1-12, Pool 2 contained fractions 13-26, and Pool 3 contained the remainder the fractions. An identical protocol was performed with non-denatured HBsAg. Fraction numbers begin at the bottom of the tube (the highest density and largest aggregates), and three equivalent pools were established: fractions 1-3 for Pool 1A, fractions 4-18 for Pool 2A, and the remainder of the fractions for Pool 3A. The volumes were adjusted to contain 2.5 mg per ml of protein and the samples were then used to determine whether the denatured HBsAg was capable of inducing oral tolerance as opposed to the native protein, which instead had boosted a preexisting immunity. CB 17 mice were immunized with native HBsAg in CFA on day 28, and boosted intraperitoneally on day 14. Some animals were then fed PBS (control), or 0.1, 1, or 3 mg of the denatured HBsAg for four consecutive doses every second day. Spleen cells were isolated and cultured with the addition of PBS, or 0.1, 1 or 5 μg of native HBsAg for 3 days. All cultures were pulsed with tritiated thymidine for 18 hours, and the amount of incorporated label was determined by scintillography. As illustrated in Fig. 6, the dissociated HBsAg induced tolerance following oral administration while the mock-dissociated protein did not.

This experiment illustrates that denaturation of the HBsAg resulted in minimally in the loss of the ability to act as a booster immunogen when presented per os, and showed statistically significant immune suppression only when 5 mg of the smallest MW fraction was fed.

Example 6: Determination of the average aggregate size maximally effective in inducing mucosal tolerance.

To determine the approximate molecular weight of aggregates present in the three pools, an aliquot of HBsAg was treated as described above, and applied to a Sepharose G200 column. This size filtration resin can resolve molecular weights from approximately 20kD (approximately 3 HBsAg monomer aggregates) to 200 kD (approximately 200 HBsAg monomer aggregates). The OD_{280nm} of the effluent was monitored, and average size of HBsAg aggregates present in the pools calculated. Pool 1 was determined to elute predominantly in the void volume indicating an average size of greater than 2,000 kD. Pool 2 (low mw denatured HBcAg), a fraction determined to have demonstrable but less than maximal effectiveness, had an M_R of 165 kD, or approximately 17 subunits, suggesting that the genetic engineering successfully prevented oligomer formation. Figure 8 shows that the size of the engineered antigen (HBeAg) was significantly smaller than native as determined by sedimentation on a sucrose density gradient. Pool 3 was composed predominantly of aggregates of between 10 and 3 monomers.

Example 7: Construction expression, isolation and effectiveness of modified HBeAg

The experiments described above clearly indicate that the ability of an antigen to induce mucosal tolerance depends critically on its physical conformation and thus physical size. Proteins which self-assemble into macromolecular structures such as HBsAg and HBcAg expressed in transgenic systems resulting in pseudovirions and pseudocapsids with multiple-nanometer diameters either fail to induce mucosal tolerance (HBcAg) or stimulate a booster effect in animals previously rendered immune by other means. To establish this principle for an application for treating certain chronic infections as described in U.S. Patent No. 6,355,348, the gene (residues 1816 through 2454 with modifications) encoding HBcAg (serotype ayw) was modified to a) eliminate its capacity to bind nucleic acids through the protamine domain entailing the arginine rich 5' section of the gene, and b) to minimize the propensity of the protein to self-assemble

into pseudocapsids, a property mapped by others to this coding region (Schodel et al., 1993, JBC 268:1332-1337).

Plasmid TKHH2 (Will et. al., 1985, PNAS 82:800-895) containing a tandem head to tail copy of HBV genome was digested with restriction endonucleases to produce the desired coding sequence, and inserted into a pET28A vector system (NovaGen). Following transfection into competent B-21 E. coli, successful transformants were selected by growth on EMB agar containing 50 μg/ml ampicillin. Following dilution in LB medium containing ampicillin, bacteria were re-streaked and a single colony selected for seed-stock expansion. Seed stock was established in 50% glycerol and 50% LB medium stored at –80°C. The amino-terminus of the protein was altered to contain an additional 7 amino acids from the native pre-core sequence to enhance solubility. The carboxy- terminus contains two amino acids (leu, glu) as an artifact of engineering and a 6-histidine tail for protein isolation by nickel affinity. The plasmid engineered for protein expression was sequenced and the DNA sequence was translated into the following amino acid composition. The plasmid DNA sequence had an exact 477 nucleotide overlap with the published DNA of the HBV genome, and when translated to preferred amino acid usage in E. coli, resulted in the following peptide sequence (SEQ ID NO:3):

MQASKLCLGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREA
LESPECHSPHHTALRQAILCWGELMTLATWVGVNLEDPASRDLVVSYVNTNMG
LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTLEHH
HHHH.

This protein differs from naturally occurring HBeAg in that five amino acid residues are uniquely present in the carboxy-terminal region of the construct.

The above protein was expressed and isolated with starter culture is established in LB broth containing both ampicillin and chloramphenoicol. After overnight growth, an aliquot of the culture was used to inoculate a 20 L culture in a Micros 30 fermentor. Once the OD_{600nm} of the culture reached 8 (± 2) , the IPTG concentration was brought to 1 mM for transgene expression induction, which continued

for 4 hours. Bacterial cells were pelleted with centrifugation, resuspended in 200 mM Tris, pH 8.0, and disrupted using 4 passes though a micro-fluidizer. The lysate was stored frozen at -80°C. The lysate (100 ml) was then thawed, diluted with 200 ml 50 mM Tris pH 8.0, and centrifuged for 30 minutes at 5000 RPM. The supernatant was removed, and an additional 200 ml 50 mM Tris pH 8.0 was added. The suspension was again centrifuged at 13000 RPM for 30 min, and the supernatant then removed. The pellets were combined and solubilized with 235 ml of 8M urea in 50 mM Tris pH 8.0 with constant mixing at room temperature. After a 30 minute incubation with frequent mixing, the mixture was applied to a 1 cm x 100 cm column previously loaded with HISBIND resin (NovaGen) equilibrated with 8 M urea, 50 mM Tris pH 8.0. The flow through fraction was collected, and a wash volume (300 ml) of 8M urea in 50 mM Tris pH 8.0 passed over the column, with 50 ml fractions collected. HBeAg elution was performed by exchanging 8M urea in 50 mM Tris pH 8.0 for 1 M imidazole, eluting the bound product, and collecting 50 ml fractions. Fractions containing $\geq$ 90% product as determined by PAGE and western blotting were pooled and dialyzed into PBS. Each fraction of affinity-column eluate was assayed by both PAGE, stained using Coomassie dye or silver-staining techniques, and by western blotting. Densitometric analysis of the resulting gel was performed using each aliquot, and only samples of greater than 90% purity were pooled for subsequent use. Proteins other than transgene-specific bands, as determined by western blot analysis, are believed to be of BL-21 origin.

The relative efficacy of the HBeAg to induce mucosal tolerance in immune mice compared to native HBcAg or ag HBcAg was tested by first immunizing normal CB17 mice of both sexes with sub-q injections of 10 μg of native (non-modified) protein emulsified in CFA. Two weeks later, mice were boosted with intraperitoneal injections of 10 μg of the same antigen. Some mice were treated with injections of PBS/CFA and boosted with PBS to serve as non-immune controls. Selected mice received per os treatments of 0.1, 1 and 5 mg of the modified HBcAg, or doses of native HBcAg or ag HBcAg previously found to be maximally effective. Some mice received PBS per os as appropriate for a total of four treatments applied every second day. Two days following the final treatment, the mice were euthanized, and the isolated spleen cells challenged in culture with antigen in vitro. The responses were quantified by the

incorporation of ^{3}H-Tdr as described.

Figure 7 clearly shows that aggregated HBcAg was effective at inducing mucosal tolerance as previously shown. Native HBcAg appeared to be less effective, perhaps due its particulate conformation. Modified HBeAg was significantly more effective than the other two forms of the protein, while HBeAg administered at the same doses induced a significantly greater suppression. The suppression was greater than that observed for pool 2 (low mw denatured HBcAg), suggesting that the genetic engineering successfully prevented oligomers formation.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of inducing a systemic immune response to a peptide in a mammal, said method comprising transmucosally administering to the mammal a macromolecular aggregate of the peptide, thereby inducing a systemic immune response.

2. The method of claim 1, wherein the macromolecular aggregate comprises at least 10 peptide subunits.

3. The method of claim 2, wherein the macromolecular aggregate comprises at least one of the characteristics selected from the group consisting of:

a). 20 peptide subunits; and

b). an aggregate of a molecular weight in excess of 1,000 kD.

4. The method of claim 1, wherein the macromolecular aggregate is at least 1 nm in diameter.

5. The method of claim 4, wherein the macromolecular aggregate is at least 5 nm in diameter.

6. The method of claim 1, wherein the macromolecular aggregate is resistant to digestive degradation.

7. The method of claim 1, wherein the macromolecular aggregate is stabilized in aggregate form by chemical treatment.

8. The method of claim 1, wherein the macromolecular aggregate is stabilized in aggregate form by recombinant protein engineering of the peptide.

9. The method of claim 8, wherein the macromolecular aggregate of the peptide is further stabilized in aggregate form by chemical treatment.

10. The method of claim 1, wherein the peptide is selected from the group consisting of a hepatitis B viral surface protein, a hepatitis B viral nucleocapsid protein, and a hepatitis B viral envelope protein.

11. A pharmaceutical composition for inducing systemic immunity in a mammal, the composition comprising a macromolecular aggregate of a peptide and a suitable carrier, in an amount sufficient to induce systemic immunity when administered to a mammal transmucosally.

12. A method of suppressing a systemic immune response to a peptide in a mammal already immune to said peptide, said method comprising transmucosally administering to the mammal a macromolecular aggregate of the peptide, thereby suppressing a systemic immune response.

13. The method of claim 12, wherein the macromolecular aggregate comprises less than 21 peptide subunits.

14. The method of claim 12, wherein the macromolecular aggregate is less than 1 nm in diameter.

15. The method of claim 12, wherein the macromolecular aggregate is resistant to digestive degradation.

16. The method of claim 12, wherein the macromolecular aggregate is stabilized in aggregate form by chemical treatment.

17. The method of claim 12, wherein the macromolecular aggregate is stabilized in aggregate form by recombinant protein engineering of the peptide.

18. The method of claim 17, wherein the macromolecular aggregate of the peptide is further stabilized in aggregate form by chemical treatment.

19. The method of claim 12, wherein the peptide is selected from the group consisting of a hepatitis B viral surface protein, a hepatitis B viral nucleocapsid protein, and a hepatitis B viral envelope protein.

20. A pharmaceutical composition for suppressing systemic immunity in a mammal, the composition comprising a molecular aggregate of a peptide and a suitable carrier, in an amount sufficient to suppress systemic immunity when administered to a mammal transmucosally.

Attorney Docket No.: **054044-5002-PR**

SOLE

DECLARATION AND POWER OF ATTORNEY

(Original Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled

TRANSMUCOSAL ADMINISTRATION OF AGGREGATED ANTIGENS

the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d), of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

FOREIGN PRIORITY APPLICATION(S)

			Priority Claimed
			[ ] Yes [ ] No
(Number)	(Country)	(Day/month/year filed)	

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional patent application(s) listed below and have also identified below any United States provisional patent application(s) having a filing date before that of the application on which priority is claimed:

PROVISIONAL PRIORITY PATENT APPLICATION(S)

Priority Claimed
[ ] Yes [ ] No

_____
(Application No.) (Filing Date)

And I hereby appoint the registered attorneys and agents associated with **MORGAN, LEWIS & BOCKIUS, L.L.P., Customer No. 028977,** as my attorneys or agents with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all correspondence to **Customer No. 028977, namely, MORGAN, LEWIS & BOCKIUS, L.L.P.,** 1701 Market Street, Philadelphia, Pennsylvania 19103. Please direct all communications and telephone calls to Kathryn Doyle, Ph.D., J.D. at (215) 963-4723.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full name of sole inventor, if any **Frank Michaels**

Inventor's Signature _____

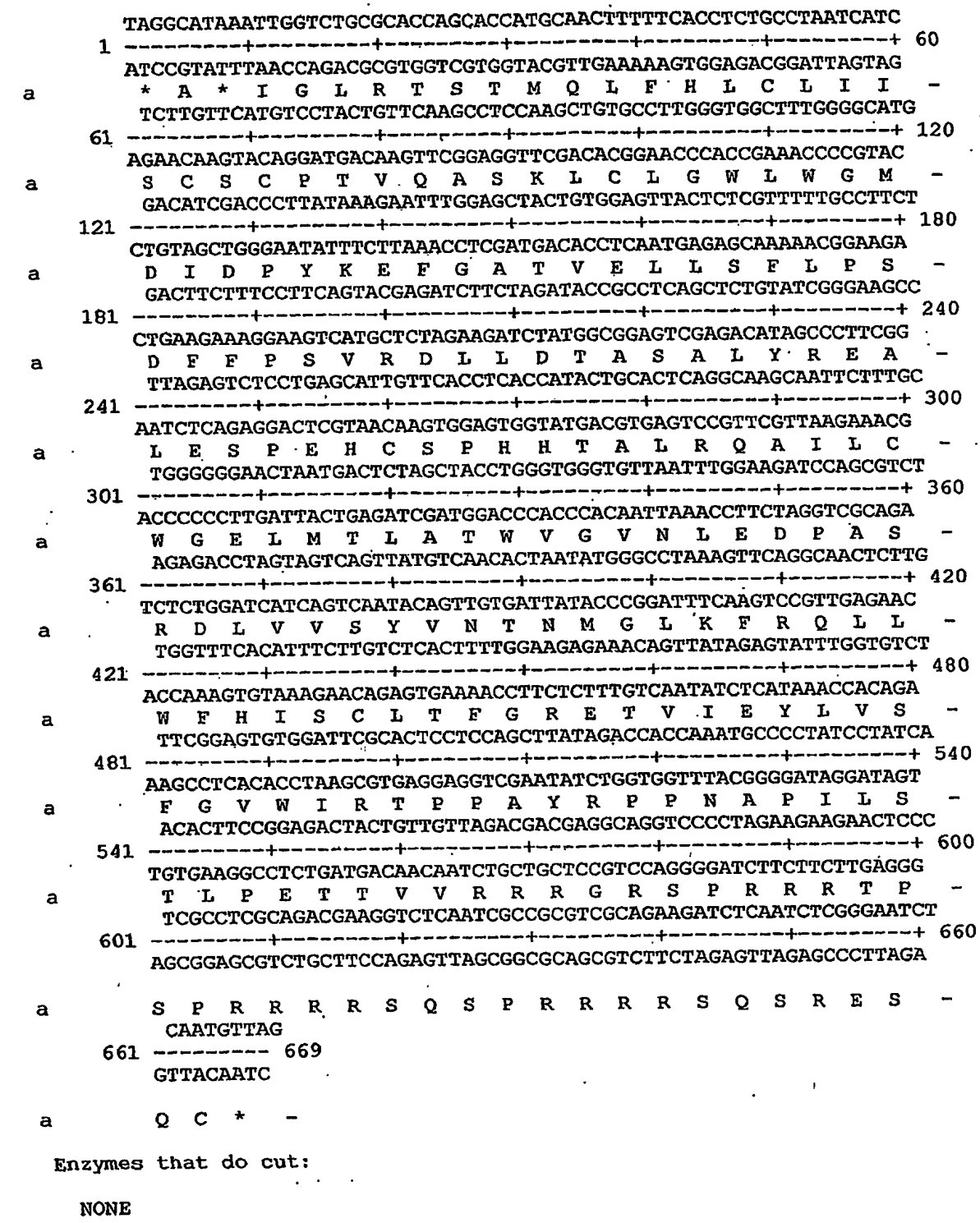
Date October 16, 2002

Residence Havertown, Pennsylvania 19083

Citizenship US

Post Office Address 1307 Virginia Avenue, Havertown, Pennsylvania 19083

Figure 1.



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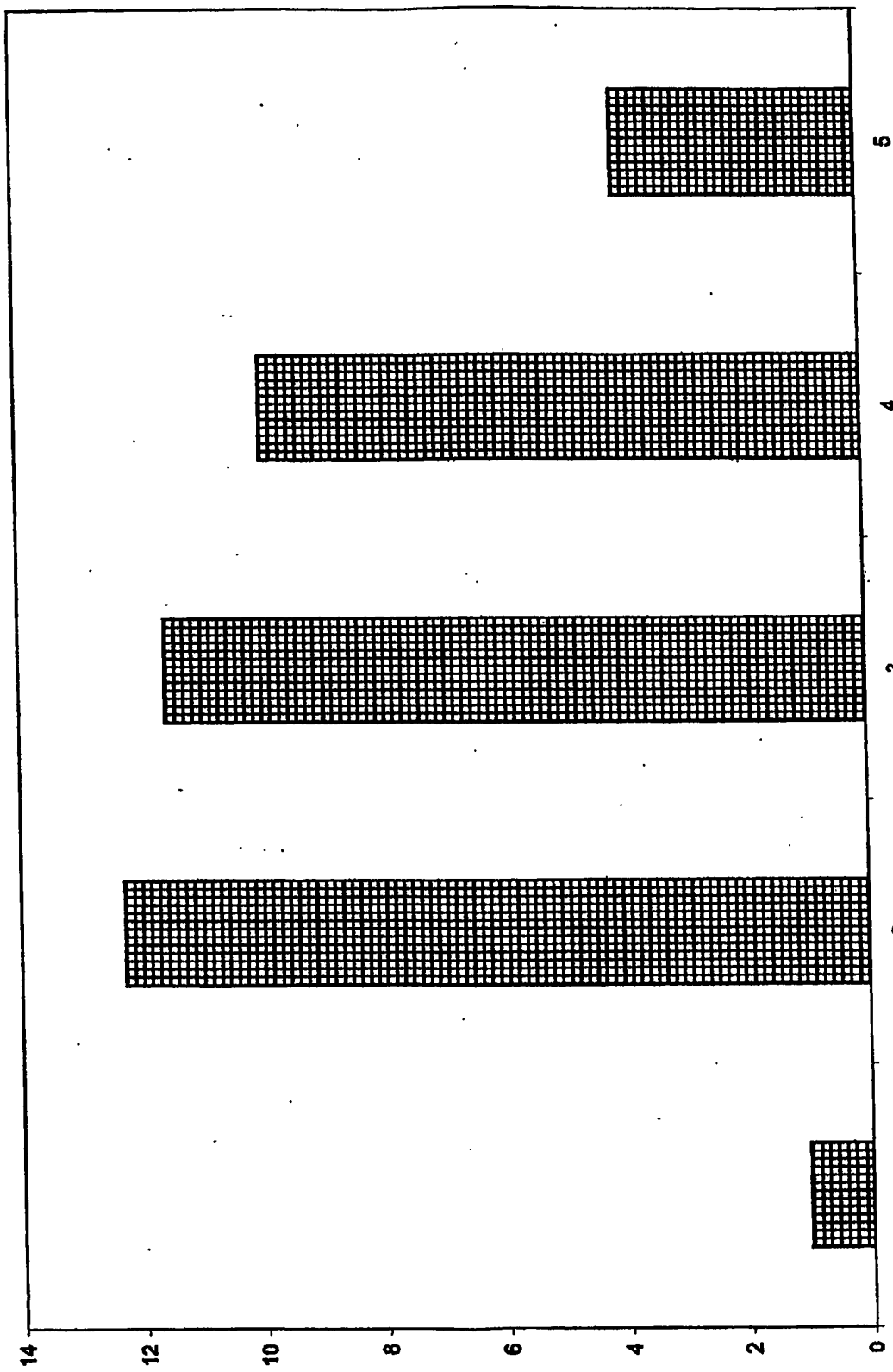

Figure 2

Figure 3

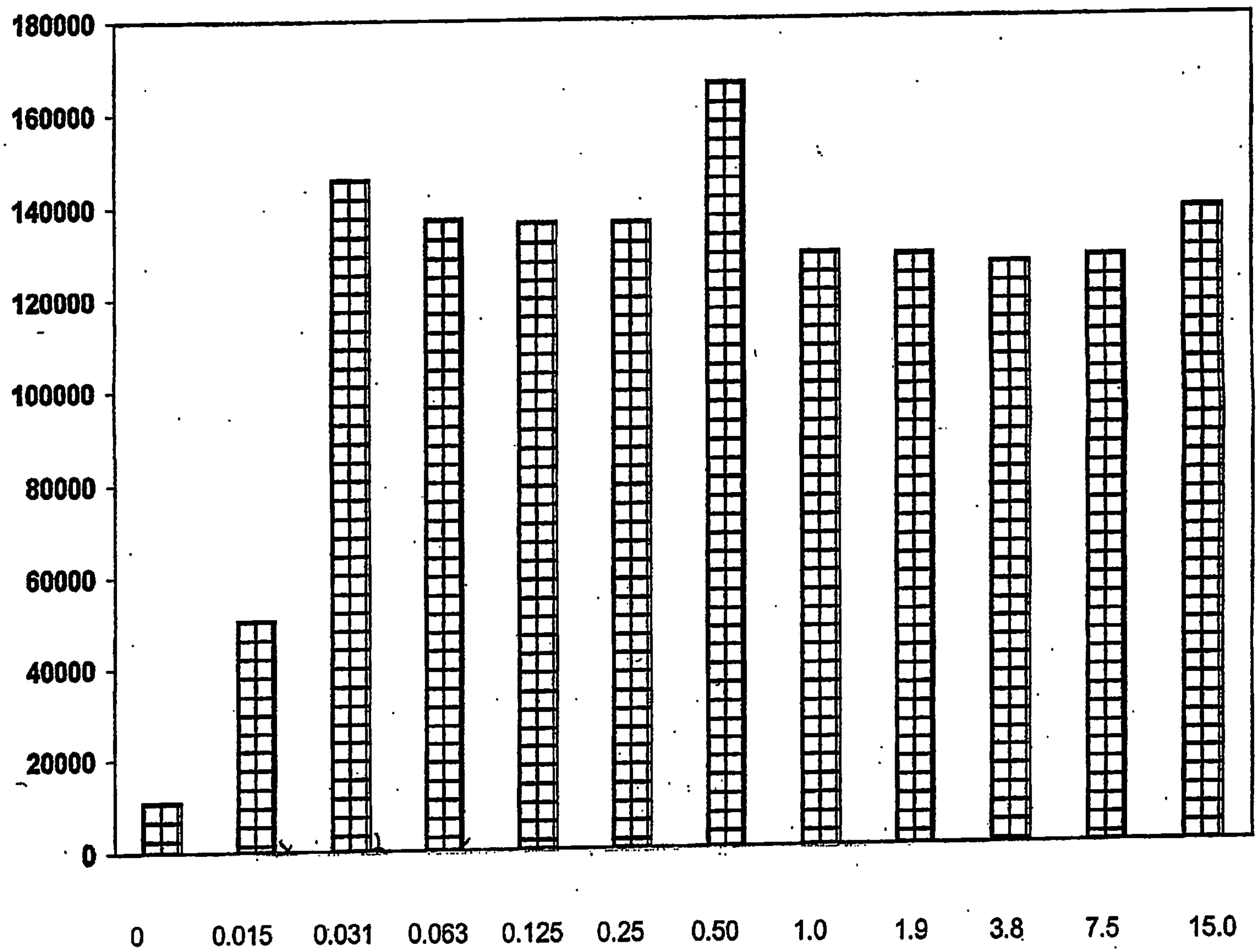
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Figure 4a

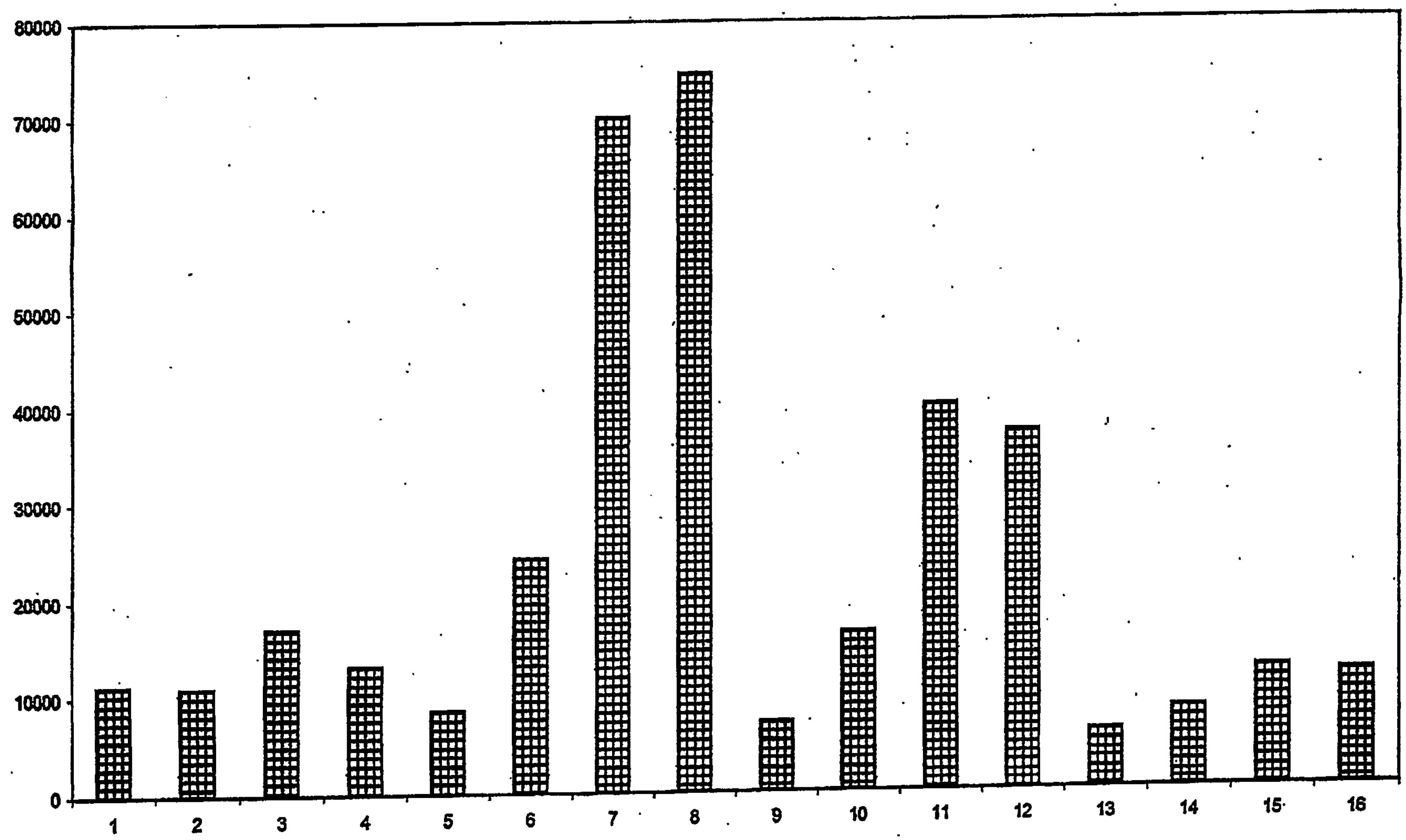

Figure 4b

Treatment	day 2	day 3	
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Imm 0.1 ug		5614	10904
Imm 1 ug		7266	16991
Imm 5 ug		5882	13120
Imm OT (3mg) no ag		4924	8409
Imm OT (3mg) 0.1 ug ag		7235	24250
Imm OT (3mg) 1 ug		14605	70078
Imm OT (3mg) 5 ag		11995	74585
Imm OT (1mg) no ag		3558	7079
Imm OT (1mg) 0.1 ug ag		4871	16339
Imm OT (1mg) 1 ug		4949	39954
Imm OT (1mg) 5 ag		4172	37059
Imm OT (0.1mg) no ag		5661	5904
Imm OT (0.1mg) 0.1 ug ag		12094	8168
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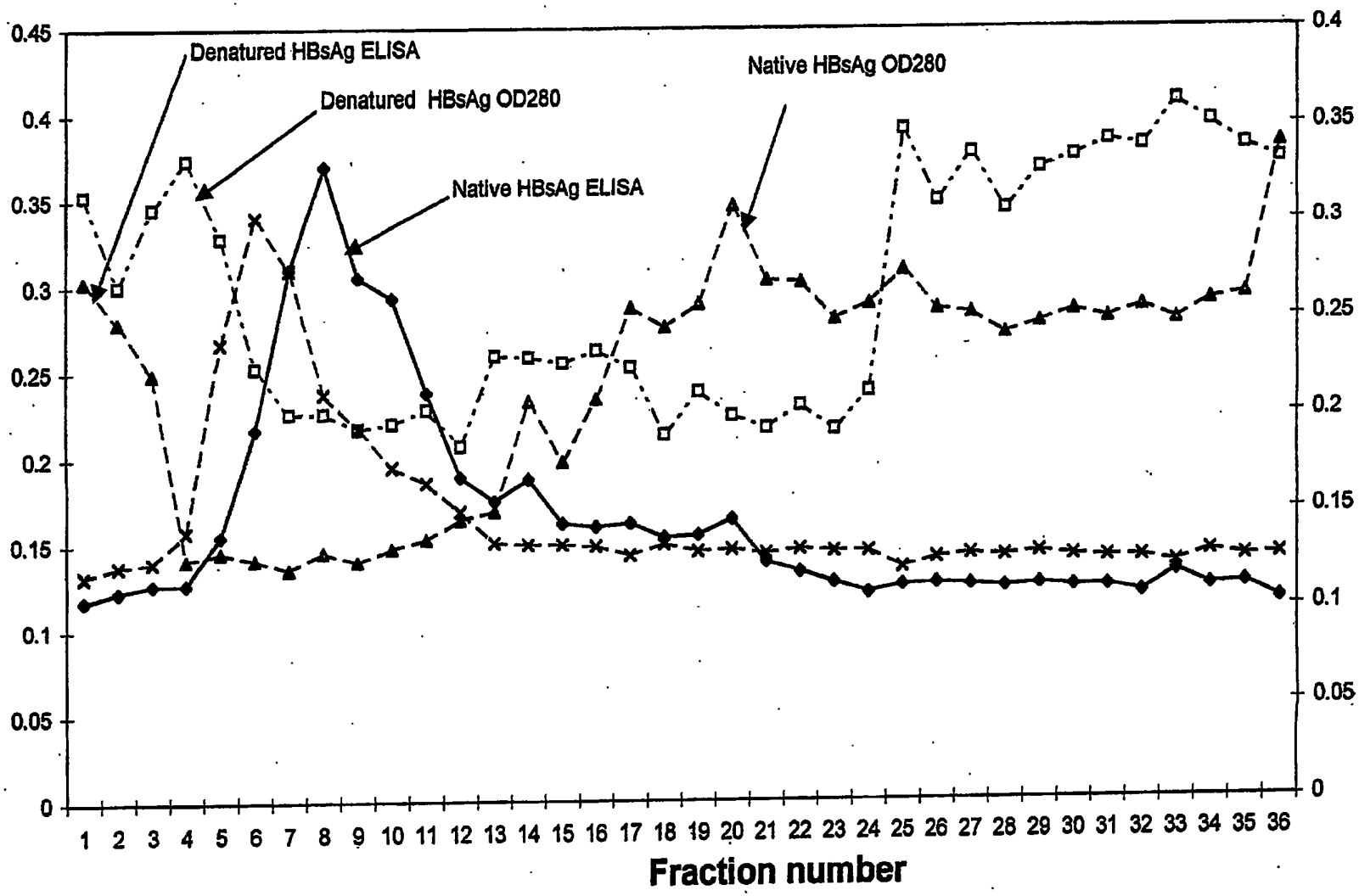
Imm OT (0.1mg) 5 ag		36710	11754

Figure 5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Native HBsAg OD280
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0.1
0.05
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0.4
0.35
0.3
0.25
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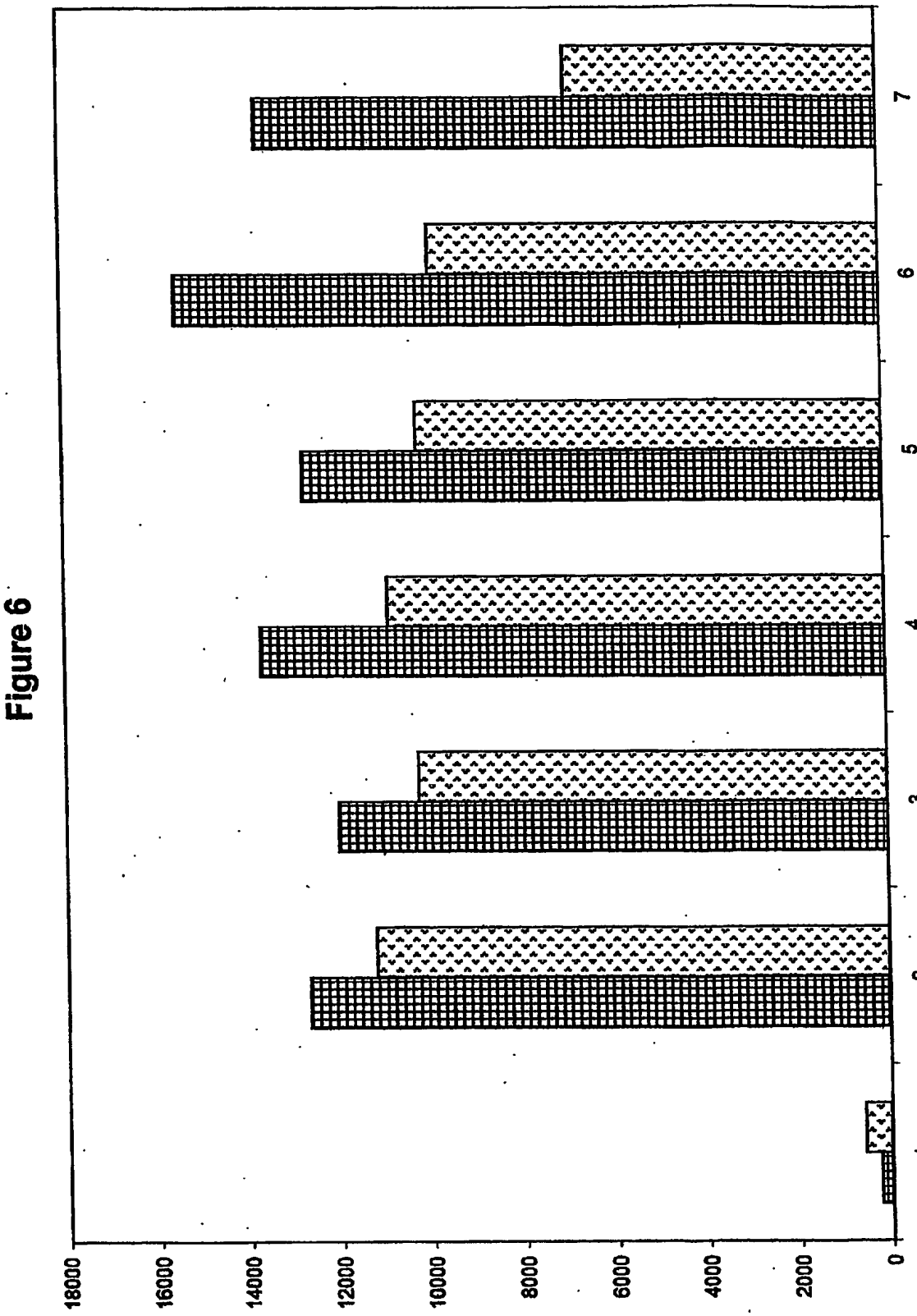

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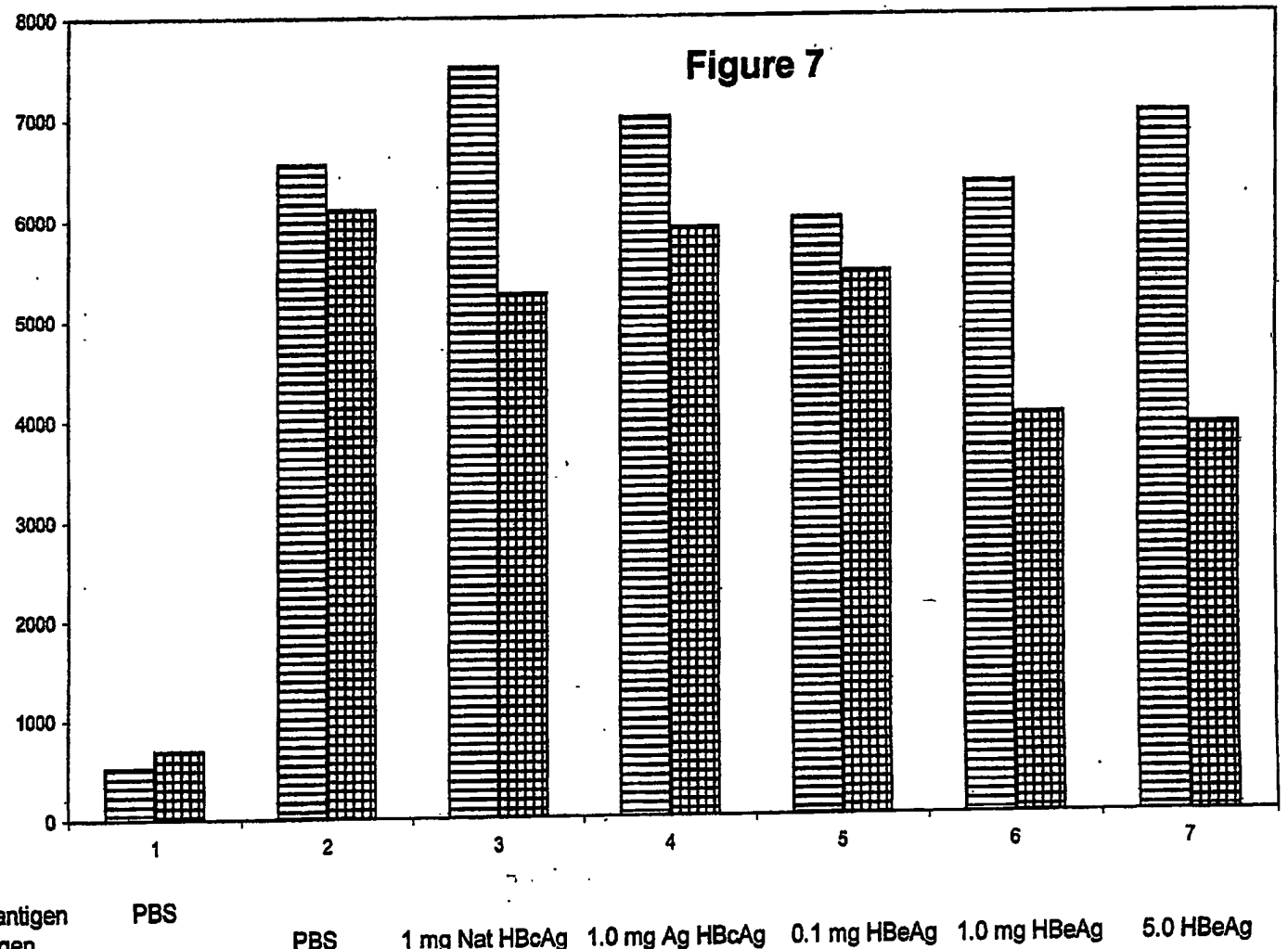
Figure 7
8000
7000
6000
5000
4000
3000
2000
1000
0
1
2
3
4
5
6
7
Immunizing antigen
Tolerizing antigen
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PBS
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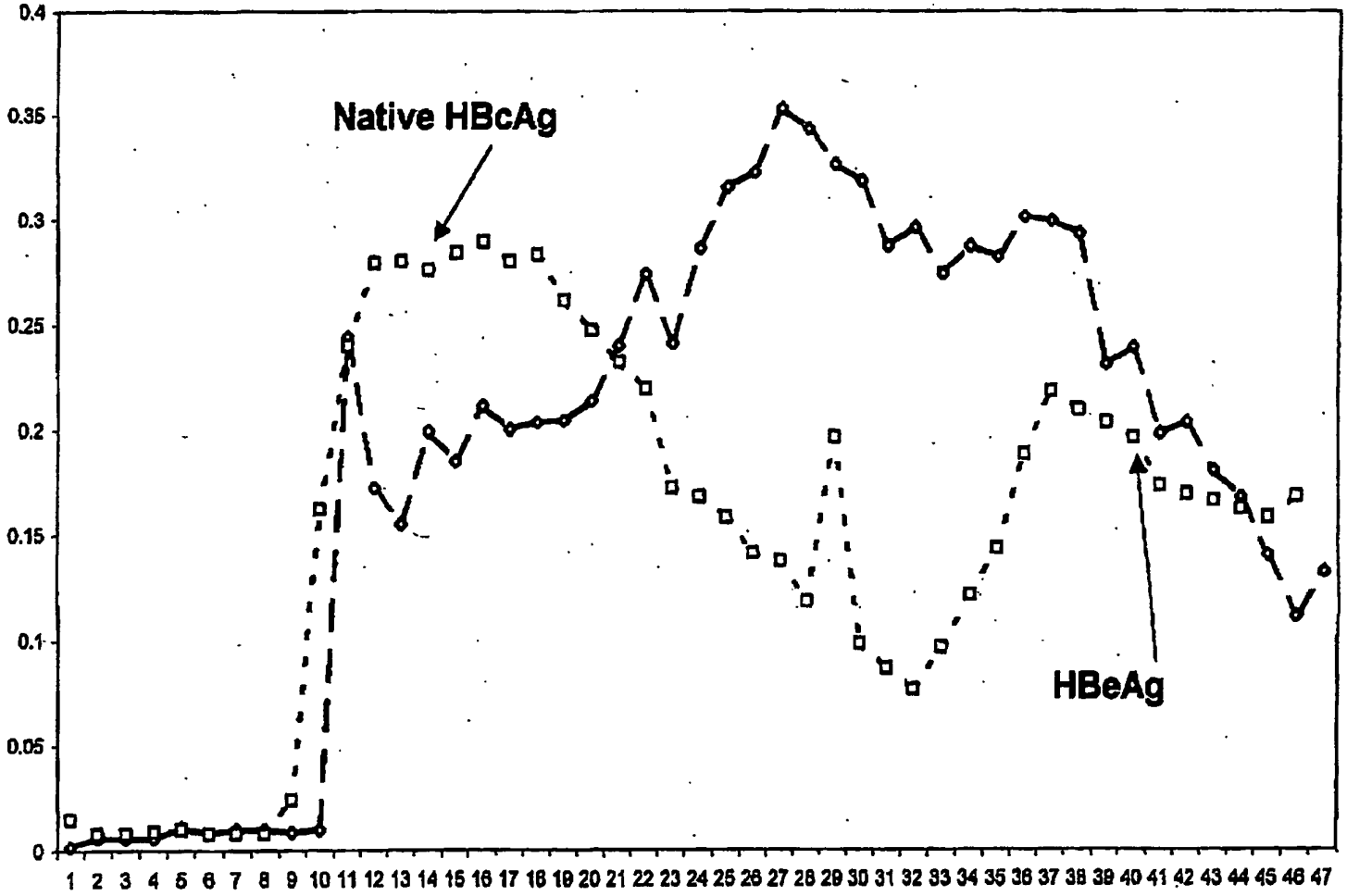
1.0 mg Ag HBcAg
0.1 mg HBeAg
1.0 mg HBeAg
5.0 HBeAg

Figure 8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tgc cta atc atc tct tgt tca tgt cct act gtt caa gcc tcc aag ctg       96
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15                  20                  25                  30

tgc ctt ggg tgg ctt tgg ggc atg gac atc gac cct tat aaa gaa ttt      144
Cys Leu Gly Trp Leu Trp Gly Met Asp Ile Asp Pro Tyr Lys Glu Phe
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tta gag tct cct gag cat tgt tca cct cac cat act gca ctc agg caa      288
Leu Glu Ser Pro Glu His Cys Ser Pro His His Thr Ala Leu Arg Gln
    80                  85                  90

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Phe Gly Val Trp Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala
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cct atc cta tca aca ctt ccg gag act act gtt gtt aga cga cga ggc      576
Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr Val Val Arg Arg Arg Gly
175                 180                 185                 190

agg tcc cct aga aga aga act ccc tcg cct cgc aga cga agg tct caa      624
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Trp Leu Trp Gly Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr
        35                  40                  45

Val Glu Leu Leu Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg
    50                  55                  60

Asp Leu Leu Asp Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser
65                  70                  75                  80

Pro Glu His Cys Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu
                85                  90                  95

Cys Trp Gly Glu Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu
            100                 105                 110

Glu Asp Pro Ala Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn
        115                 120                 125

Met Gly Leu Lys Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu
    130                 135                 140

Thr Phe Gly Arg Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val
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            20                  25                  30

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser
        35                  40                  45

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu Cys His Ser Pro His
    50                  55                  60

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr
65                  70                  75                  80

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp
                85                  90                  95

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln
            100                 105                 110

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val
        115                 120                 125

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala
    130                 135                 140

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr
145                 150                 155                 160

Leu Glu His His His His His His
                165
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